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14. ABSTRACT We hypothesized that MUC1, a transmembrane glycoprotein that is overexpressed in >80% of pancreatic ductal adenocarcinoma induces a pro-angiogenic tumor microenvironment by increasing the level of NRP-1 and VEGF thereby enhancing angiogenesis and metastasis. We report that MUC1^{hi} PC cells and tumors *in vitro* and *in vivo* not only express higher levels of NRP1 but also express higher levels of VEGFR2, pVEGFR2^{1175}, pVEGFR2^{996}, and VEGFR3 as well as secrete higher levels of VEGF than MUC1^{low} PC cells. This enables the MUC1^{hi}/NRP^{hi} cells to generate long ectopic blood vessels and enhanced distant metastasis. In the proposal, we also hypothesized that blocking the interaction between VEGF$_{165}$ and NRP-1 within the tumor microenvironment will lead to therapeutic benefit. Indeed, *in vivo* blocking NRP1 significantly reduces tumor burden in the MUC1^{hi} tumors. In fact 4 out of 7 mice had a complete response, 2 had small tumors and 1 mouse did not respond and developed large tumor. This treatment had no significant effect on the MUC1^{low} tumors. This is not surprising since MUC1^{low} tumors have very low levels of NRP1 and VEGFR2. It should be noted that these MUC1^{low} tumors do however grow but may eventually form stable disease. Thus, we conclude that NRP1 may be a promising target for MUC1^{hi} PC and in the future conjugating to the MUC1 antibody for targeted delivery may enhance its potency.

15. SUBJECT TERMS
Neuropilin-1, MUC1, Angiogenesis, Pancreatic Cancer

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Introduction: Pancreatic cancer is a lethal disease. We hypothesize that MUC1 induces a pro-angiogenic tumor microenvironment by increasing the level of NRP-1, thus enhancing angiogenesis, disease progression and metastases. Second, blocking the interaction between VEGF$_{165}$ and NRP-1 within the tumor microenvironment will lead to therapeutic benefit. We report progress for all three tasks that we had proposed. I have thus broken the report down by tasks. The objective and specific aims are stated below. This is followed by the individual tasks with the progress made for that task. We show 11 figures in total. Figures 1-3 are for Task 1, Figures 4-7 are for Task 2, and Figures 8-11 are for Task 3.

Objectives/Specific Aims:

To test the effects of MUC1+ve PC cells on *in vitro* endothelial cell function in an NPR-1-dependent fashion.

To determine if MUC1 up-regulates NRP-1 and creates a pro-angiogenic niche *in vivo.*

To directly target angiogenesis within the tumor microenvironment by using TAB 004 MUC1 antibody conjugated to a peptide blocking VEGF-NPR-1 binding.

Task 1:

1) To test the effects of MUC1$^+$ PC cells on *in vitro* endothelial cell function in an NRP-1-dependent fashion **(0-9 months).** For this task we perform *in vitro* angiogenesis assays to assess the ability of MUC1-expressing pancreatic cells to enhance endothelial cell proliferation, invasion and tube formation via NRP-1

Thus, we first determined if a correlation exists between in MUC1 and NRP1 in a panel of human PDA cell lines that endogenously express high or low MUC1. Next we determined if MUC1 regulates NRP1 expression by conducting gain of function (stably transfected full length MUC1 in MUC1-low cells) and loss of function studies (by knocking down MUC1 using MUC1 specific siRNA). Figure 1A clearly shows that cells expressing high endogenous MUC1 also have high NRP1 while cells with low endogenous MUC1 has low NRP with a few exceptions (Su86.86 and Panc1). We show that overexpressing full length MUC1 in two separate MUC1low cell lines (BxPC3 and Panc) can induce higher expression of NRP1 (Figure 1B) while knocking down MUC1 from two other MUC1hi cell lines (CFPAC and HPAFII) can reduce NRP1 expression. Whether this regulation is direct or indirect is not yet delineated.

Next we studied whether the MUC1hi/NRP1hi cells affects formation of ectopic blood vessel (number and length) in a zebra fish embryo model of angiogenesis [1] [2]. We show for the first time that human PDA cell line BxPC3.MUC1 induces significantly more and longer vessels *in vivo* in a zebra fish embryo model than its counterpart BxPC3.Neo cells (Figure 2A-C). Furthermore, we show that BxPC3.MUC1 tumors are highly invasive and form significantly more metastasis compared to BxPC3.Neo tumors (Figure 3A and B) clearly suggesting that the tumors with high MUC1 can enhance proliferation by affecting vessel formation. We will confirm this result in an *in vitro* tube formation assay. However we believe that the in vivo assay is more relevant and does not depend on the matrigel and the type of endothelial cells used.

Figure 1: High MUC1 expressing PC cells have high expression of NRP1 A) T3M4, BxPC3.MUC1, Capan2, CFPAC, HPAFII, and HPAC express moderate/high levels of MUC1. These MUC1-hi cells all express moderate to high levels of NRP1. Capan1, MiaPaca2, BxPC3, Su86.86, HS766T, Panc1, and Panc2 cells have low/intermediate levels of MUC1. Other than Su86.96 and Panc1, these low MUC1 cells also express low levels of NRP1. B) When BxPC3 and Panc02 cells were stably transfected with full length MUC1, NRP 1 expression was substantially increased. C) Similarly, when MUC1 was down regulated in HPAC and CFPAC cells using siRNA, NRP1 levels were significantly downregulated. B-actin served as control for equal loading of protein.
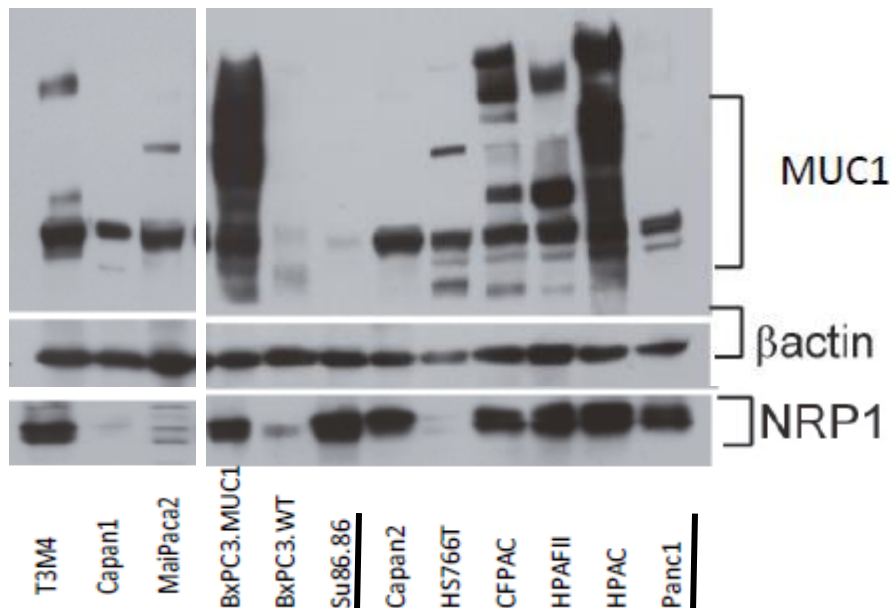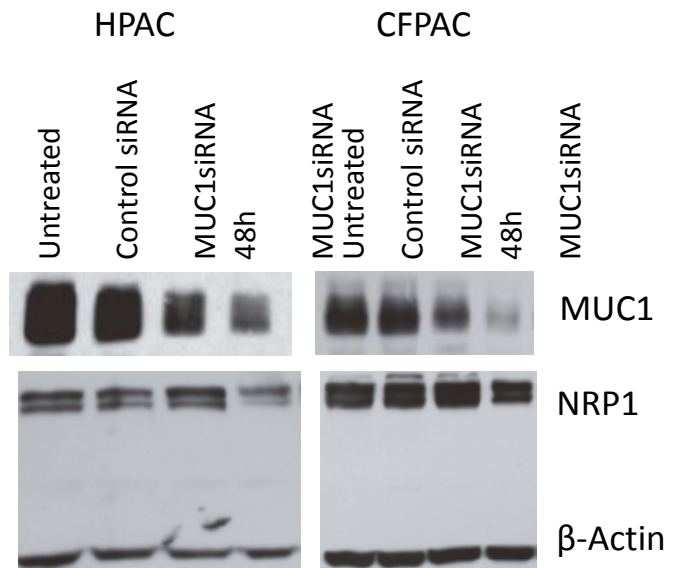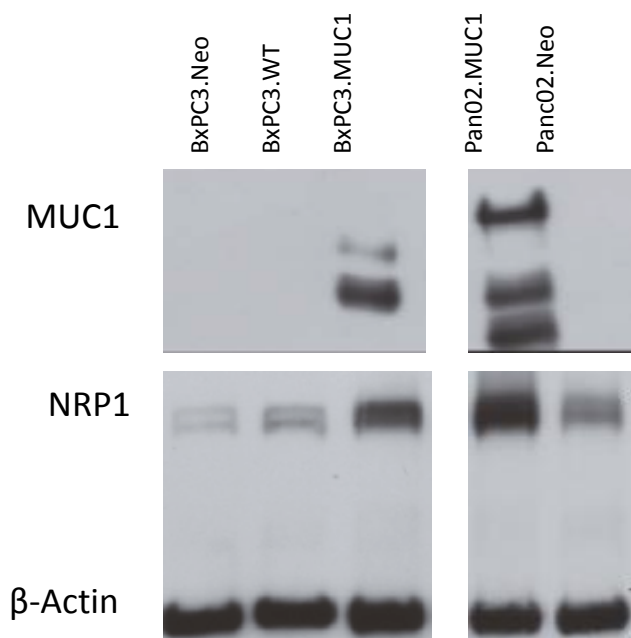
Figure 2: MUC1 expressing BxPC3 induced tumor angiogenesis

in zebrafish tumor xenograft model. A) Representative image of zebrafish embryo with formation of ectopic blood vessels. B. Quantitative measurement of number of ectopic vessels formed in the embryo C. Quantitative measurement of the length of the ectopic vessels formed in the embryo (n=100 embryos each). t test*p=0.005 between BxPC3.Neo and BxPC3.MUC1.

A.

| Ctrl-No cells | BXPC3-Neo | BXPC3-MUC1 |
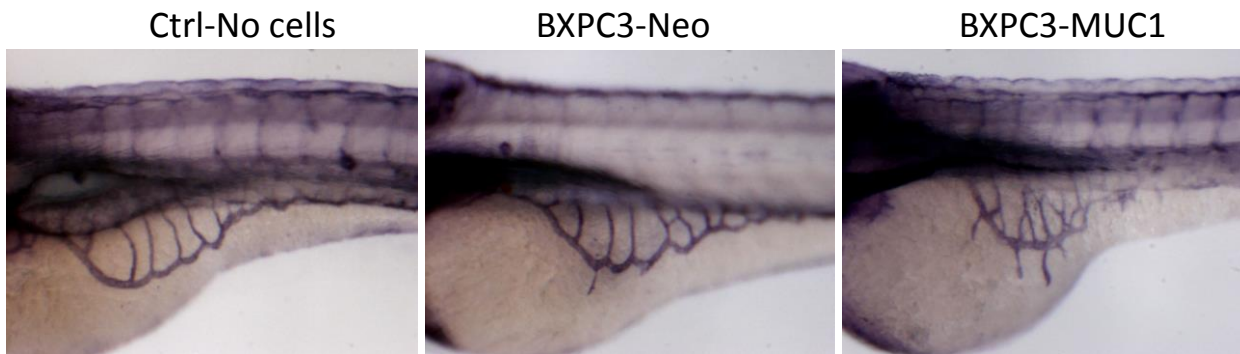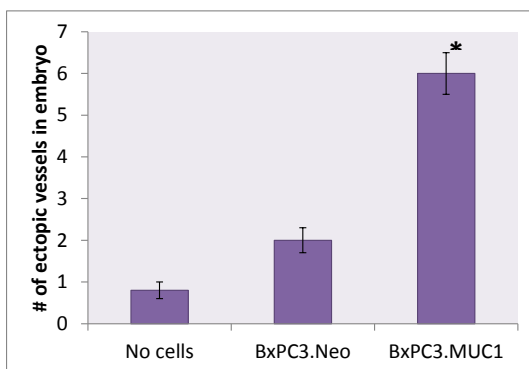
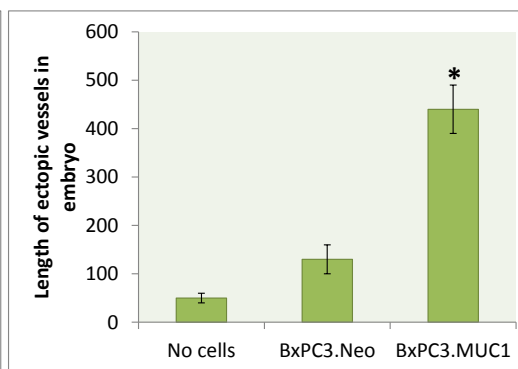**B.**   **C.**

Figure 3: MUC1 overexpression in BxPC3 promotes tumor metastasis in zebrafish/tumor xenograft. A. Represenetative image of zebra fish embryo with metastatic lesions. B. Quantitative measurement of % metastasis in xenografted tumor cells. Table shows number of embryos and the significance.

A.

No Metastasis with xenograft

Metastasis with BxPC3-Neo xenograft

Metastasis with BxPC3-MUC1 xenograft

B.

Cell Line	%Metastasis	SEM	3-group total embryos
BxPC3-Neo	5.71%	2.97%	88
BxPC3-Muc1	31.28%	4.36%	104

t-test (Neo vs. Muc1)   0.0042   p≤0.01
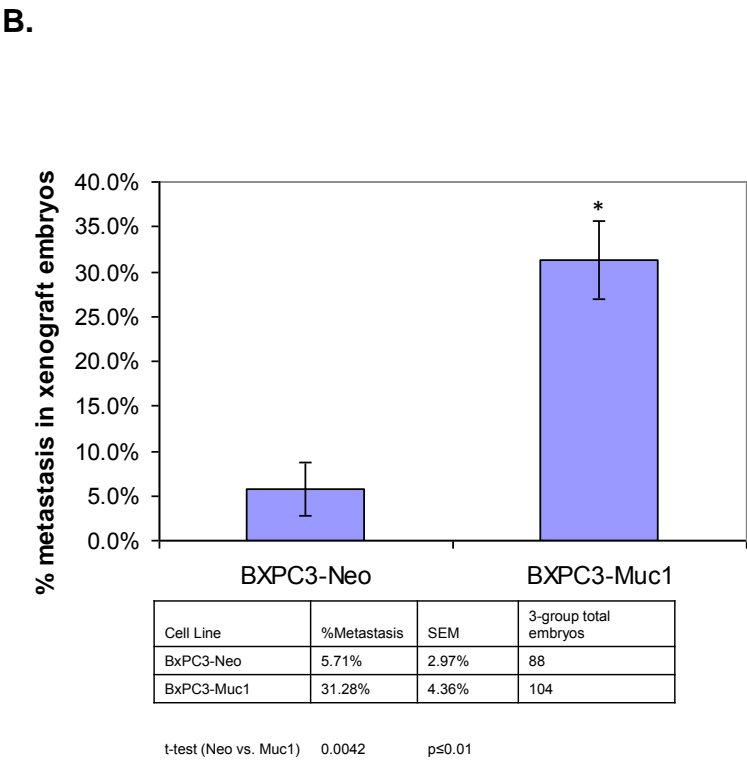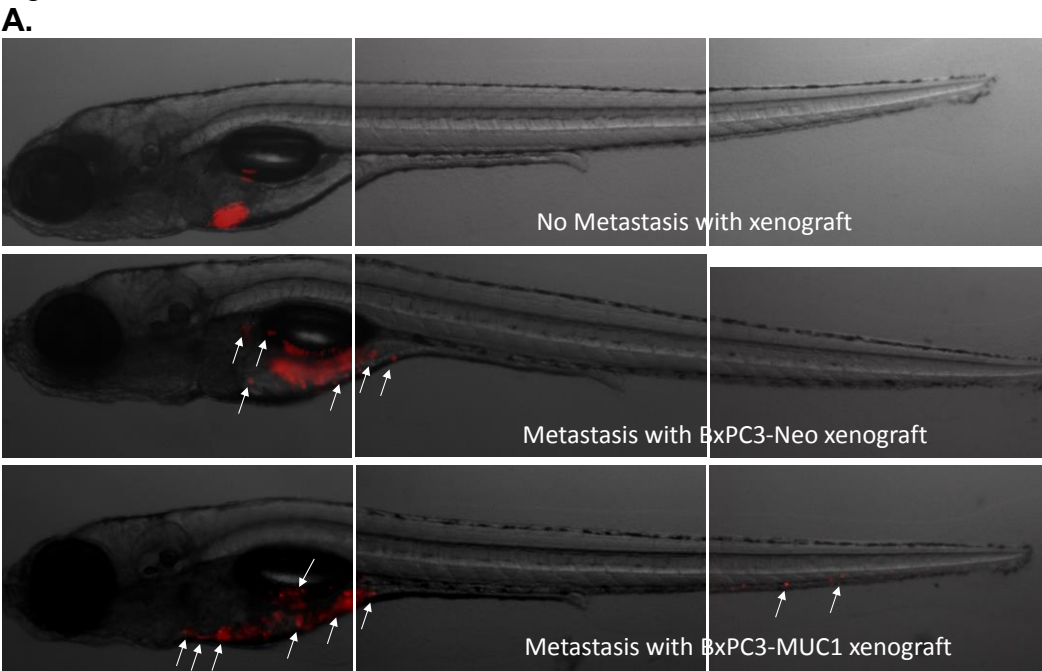
Task 2:

To determine if MUC1 up-regulates NRP-1 and creates a pro-angiogenic niche *in vivo* **(0-18 months).** For Task 2, we will use our mouse models of pancreatic cancer to determine if MUC1-expressing pancreatic tumors have enhanced intra-tumoral levels of NRP-1 and angiogenesis.

We first demonstrate that expression of NRP1 is significantly higher in the spontaneously arising PDA tumors in WT (Muc1 intact) versus PDA in Muc1 null mice (Figure 4A) [3]. Cell lines from these tumors were developed and NRP1 and Muc1 levels tested by western blotting and flow cytometry (Figure 4B and C) and confirm the data in Figure 4A. We then determined that knocking down NRP1 with specific siRNA significantly reduces proliferation only in Muc1 expressing KC cells but not in KCKO cells suggesting for the first time that Muc1-ve tumors are not dependent on NRP1 for their growth but Muc1+ve tumors are (Figure 4D and E).

To further elucidate the mechanism, we determined the other VEGF receptor levels as well as secreted VEGF levels in these cells. This is especially important as NRP1 is only a co-receptor of VEGF and signaling through VEGFR is critical for the angiogenic signaling to occur [4]. We show that KCKO (Muc1 null) cells have moderately lower levels of VEGF receptor 1, 2, and 3 than its counterpart KC cells which have an intact Muc1 (Figure 5A). Further, KC cells secrete significantly higher levels of VEGF than KCKO cells (Figure 5B). It must be noted that in human cell line BxPC3.MUC1, there was only an increase in VEGFR 2 from its counterpart BxPC3.Neo. There was no effect on levels of VEGFR1 and VEGFR3 was absent in these cells (Figure 5C). We have already shown previously that BxPC3.MUC1 cells express high levels of several pro-angiogenic factors compared to BxPC3.Neo cells [5]. Why this difference between mouse and human cells in VEGFR is not clear at this time. NRP1 levels are always lower in Muc1/MUC1 low cells compared to Muc1/MUC1hi cells.

Next we studied if supernatant from these KC and KCKO cells can differentially regulate the NRP1 levels of murine endothelial cells, 2H11. As shown in Figure 6A, 2H11 cells endogenously express high levels of NRP1 and VEGFR1 but low levels of VEGFR2 and 3 (Figure 6A). However, stimulating with supernatant had no effect on the NRP1 levels as tested by flow cytometry (Figure 6B and C). We did not test the other VEGF receptors and are planning to conduct those analyses soon. Other endothelial cell lines will also be tested similarly. Even though it might not affect NRP1 levels, it is possible that there will be differential angiogenic/oncogenic signaling. VEGFR-2, the major positive signal transducer for both physiological and pathological angiogenesis is selectively expressed on vascular endothelial cells. The binding of VEGF to its receptors induces dimerization and subsequent receptor phosphorylation, which then leads to the activation of several intracellular downstream signaling pathways promoting angiogenesis [6]. Thus we looked at the levels of VEGFR2 phosphorylation *in vivo* in KC and KCKO tumors. It was of great interest to demonstrate that not only are the levels of NRP1 and VEGFR2 significantly lower in the KCKO tumors, the phosphotylation at tyrosines 1175, and 996 are significantly low (Figure 7). This is of course because the receptor level itself is low but phosphorylation of tyrosine 996 is completely absent in KCKO tumors as compared to the KC tumors suggesting that KCKO tumors that lack MUC1 regulate NRP1 and VEGFR2 and thereby downregulate the angiogenic signaling. The question still remains whether the low receptors are due to low endothelial cells and low vessels or due to the tumor cells themselves as NRP1 and VEGFR are expressed on both the epithelial and the endothelial cells. This will be studied in great detail in the coming year. This also explains why KC cells were the only ones that responded to NRP siRNA and showed significantly lower proliferation in vitro (Figure 4E). This is significant since NRP1 binds VEGF-A$_{165}$ and only when co-expressed with VEGFR2, it enhances the binding of VEGF-A$_{165}$ to VEGFR-2 by 4- to 6-fold and results in sustained vascular permeability, inflammation, and endothelial cell migration.

Figure 4: A) *In vivo* expression of NRP1 and Muc1 from KC and KCKO tumors (spontaneously arising PDA developing in WT (intact Muc1) or Muc1 null mice respectively. KC tumors have higher NRP1 compared to KCKO tumors. B) *In vitro* expression of NRP1 in KC and KCKO cell lines. KC cells have higher expression of NRP1 than KCKO cells and expression increases with increasing confluency. C) *In vitro* expression of Muc1 by flow cytometry using CT2 antibody. KCKO is null for Muc1 and KC has high levels of Muc1. D) KC and KCKO cells were subjected to NRP1 specific siRNA and NRP1 levels determined at 48 and 72h post treatment by western blotting. E) KC cells treated with NRP-siRNA had significantly lower proliferation than control siRNA treated KC cells as determined by 3H-thymidine assay (*p<0.01). KCKO cells had no response to the peptide.
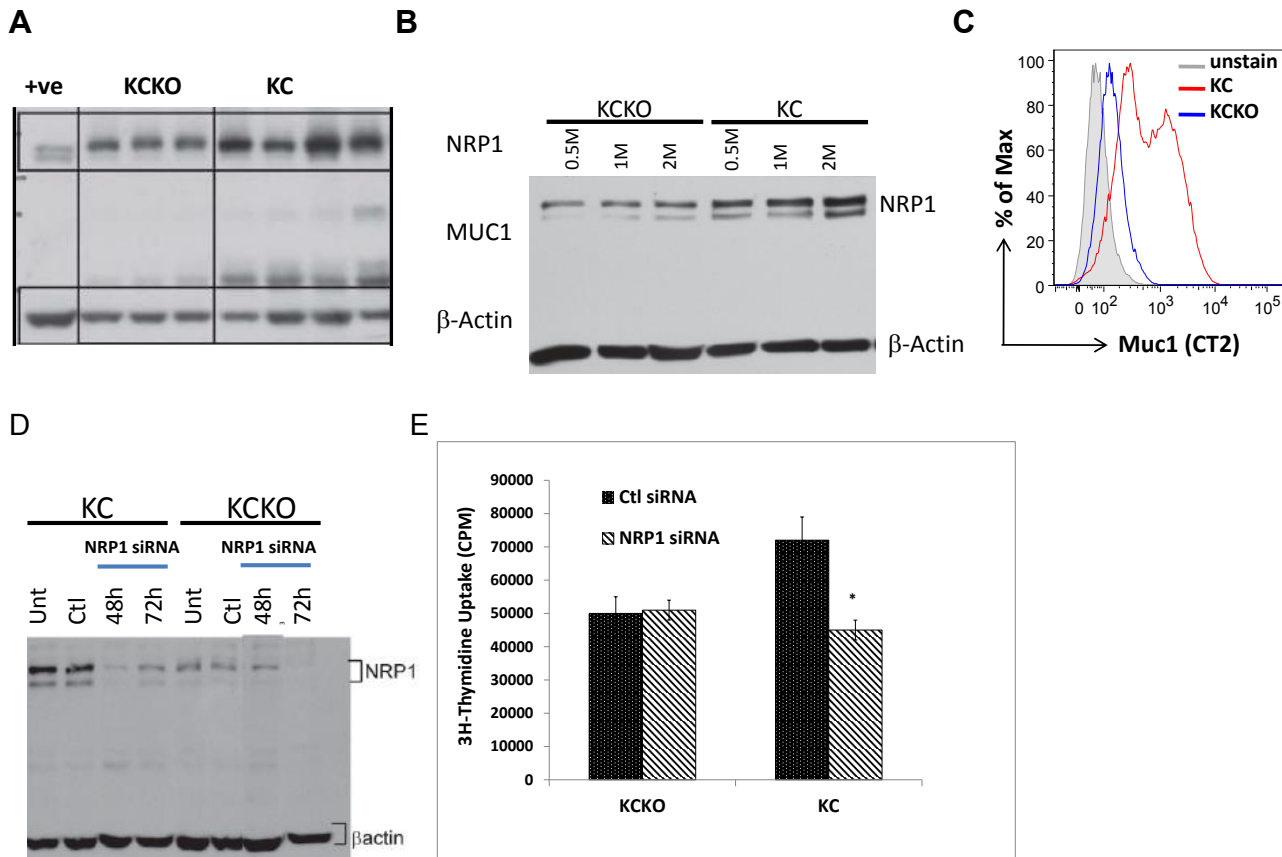
Figure 5: A) Along with NRP1, KC cells express higher levels of VEGF receptors 1, 2, and 3 and B) KC cells secrete higher levels of VEGF than KCKO cells (p<0.0001). C) BxPC3.MUC1 cells also express higher levels of VEGFR2 and NRP1. *TAB 004 and CT2 are two MUC1 monoclonal antibodies that detect the extracellular tandem repeat of MUC1 and the cytoplasmic tail respectively.* TAB 004 does not recognize mouse Muc1 and therefore not used for murine cell lines. *KC and KCKO cells were plated in medium with 10% FBS in 6-well plates overnight. Media was replaced with fresh medium without FBS. After 24hr culture, supernatants were collected and VEGF concentrations determined by ELISA.*
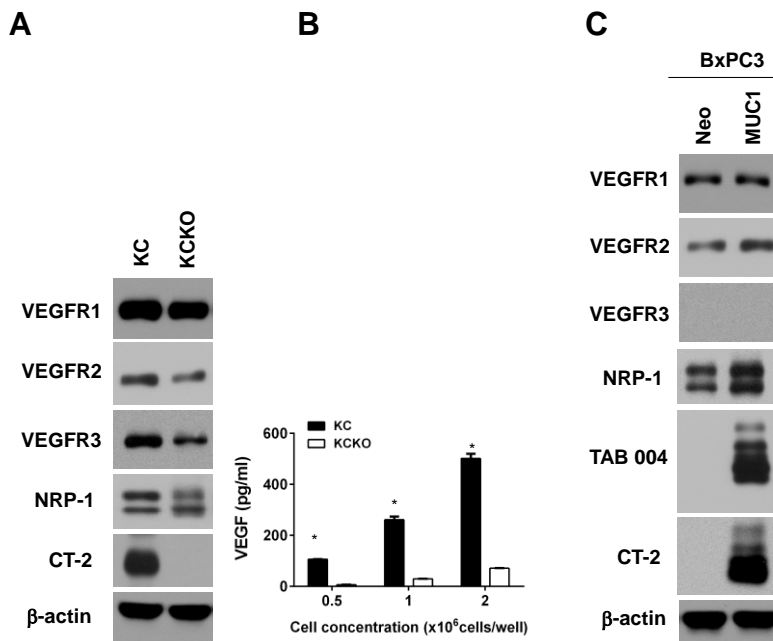

Figure 6: A) Western blot analysis of murine endothelial cell line 2H11. These cells express high levels of NRP1 and VEGFR1 B) Expression of NRP1 is confirmed by flow cytometry. C) Tumor derived conditioned medium from KC or KCKO cells did not alter levels of NRP1 in 2H11 cells as determined by flow cytometry.
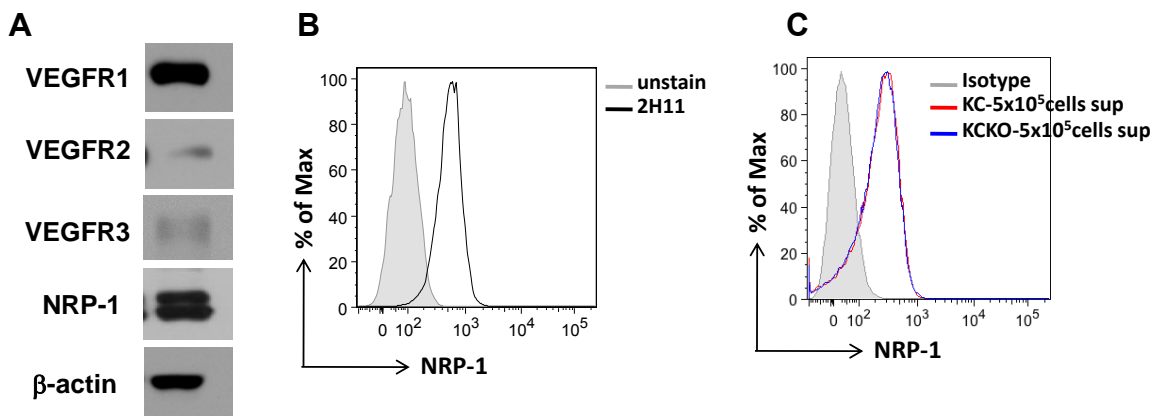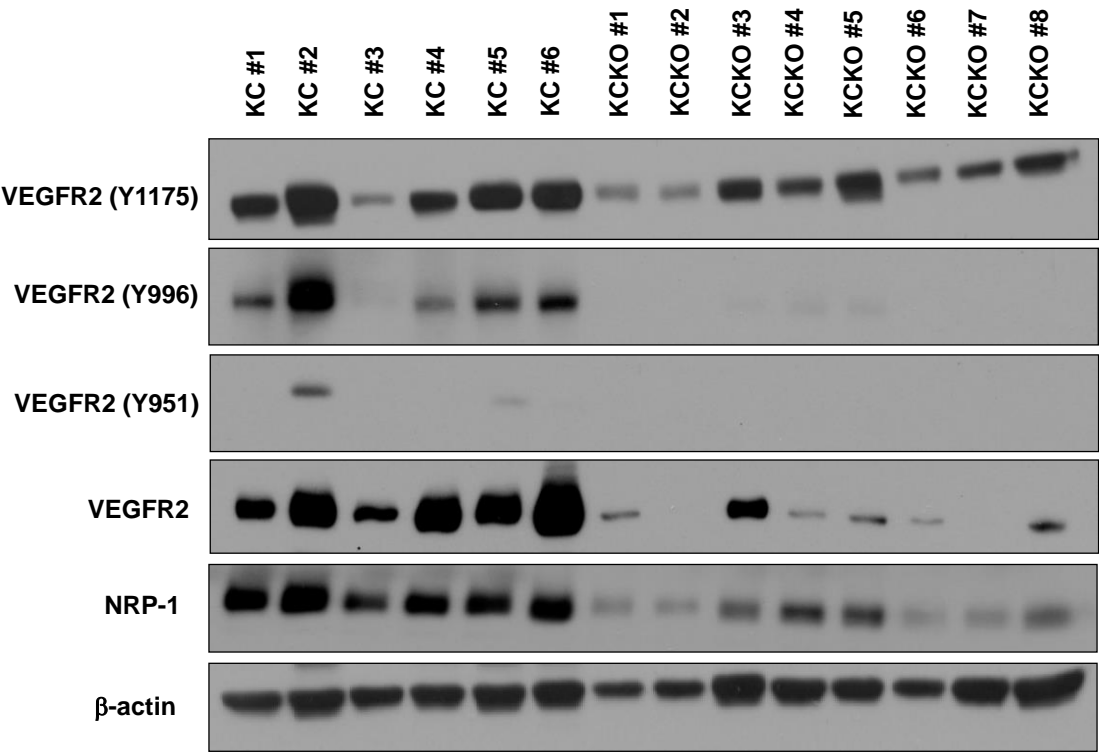
Figure 7: KCKO tumors *in vivo* display lower level of NRP1 and VEGFR2 and its phosphorylations *in vivo.* Western blotting with the indicated antibodies: N=6 KC mice and 8 KCKO mice tumors were evaluated.

Task 3: To directly target angiogenesis within the tumor microenvironment by using TAB 004 conjugated to an inhibitory peptide that blocks VEGF-NPR-1 interaction (12-24 months). In this aim, we will attempt targeted drug delivery using an antibody specific for tumor-associated MUC1 (TAB 004) conjugated to a peptide inhibitor of VEGF-NRP-1 binding.

Before we injected the cells *in vivo*, we first confirmed that BxPC3.MUC1 tumor cells proliferate at a significantly higher rate than BxPC3.Neo (Figure 8A). Next we show that the tumors *in vivo* recapitulate the *in vitro* finding that MUC1-expressing cells express more NRP1 and VEGF (Figure 8B). Finally tumor bearing mice were treated with the NRP1 blocking peptide. *Fourteen nude mice injected with 3 x10^6 BxPC3 Neo or MUC1 cells. After 2 weeks, we randomized groups and started therapy with A7R - NRP1 blocking peptide - 20mg/kg injected subcutaneously 3 times a week for 5 weeks, PBS served as the vehicle control. Mice bearing* BxPC3.MUC1 tumors responded well to the mono-therapy with significantly lower tumor burden between treated and untreated mice and with 4 out of 7 mice showing a complete response. In contrast the BxPC3.Neo tumors did not respond as well to the therapy with no significant change in the tumor burden between treated and vehicle treated mice (Figure 8).

Figure 8: Blocking with NRP blocker significantly reduced tumor growth in BxPC3.MUC1 tumors but had no effect on BxPC3.Neo tumors. A) 3H-Thymidine assay showing higher proliferation of BxPC3.MUC1 cells (t-test: * P=0.06, **p<0.05, ***p<0.01) B) Western blotting and densitometric analysis of NRP1 levels and VEGF levels *in vivo* in the BxPC3 tumor xenografts in nude mice. BxPC3.MUC1 tumors have higher levels of NRP1 and VEGF than BxPC3.Neo tumors. C) NRP-1 blocking peptide causes significant reduction in BxPC3.MUC1 tumor burden (p=0.07). Four out of seven mice showed a complete response.
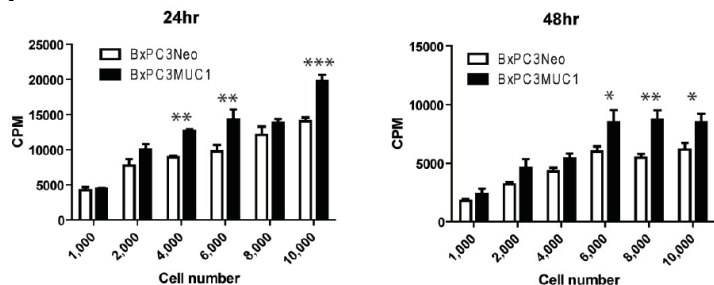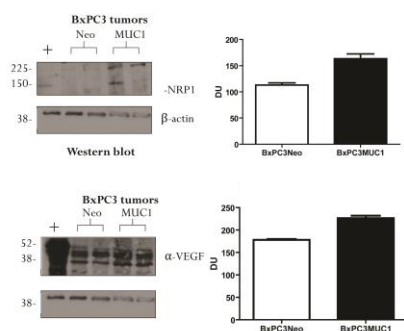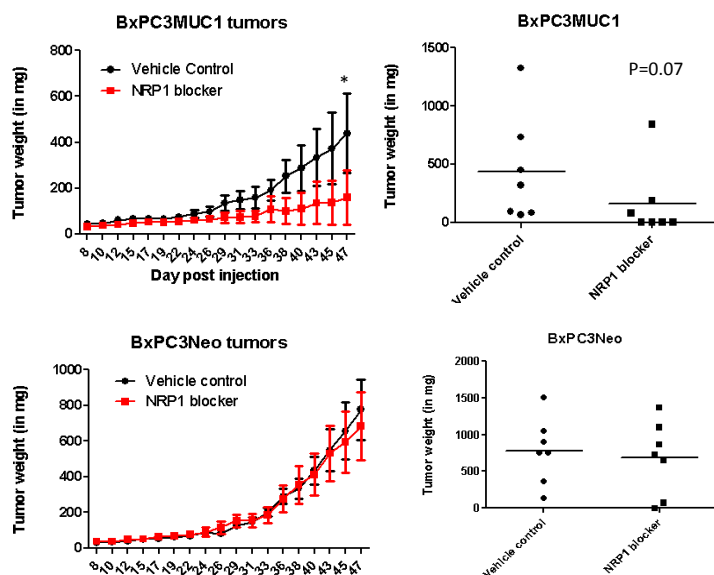
To determine what downstream factors may be altered due to the ineffective NRP1/VEGF signaling axis and contribute to the difference observed in angiogenesis between MUC1hi and low cells, we conducted a multiplex bioassay on tumor derived conditioned media. The most change was observed in Leukocyte Inhibitory Factor (LIF) (Figure 9). LIF inhibits angiogenesis in vitro, an effect that can be correlated with a LIF-mediated decrease in endothelial cell proliferation, migration and extracellular proteolysis [7]. LIF was lower both in the supernatant of BxPC3.MUC1 and HPAF cells compared to BxPC3.Neo and HPAF cells treated with MUC1siRNA. In contrast, MMP2 was higher in the BxPC3.MUC1 cell supernatant. This data is significant as it is the first indication of LIF being involved in tumor progression and angiogenesis in pancreatic cancer.

Figure 9: Multiplex analysis of the supernatants showed decreased LIF and increased MMP2 in BxPC3.MUC1 versus BxPC3.Neo cells. Similar trend was seen in HPAF cells treated with control and MUC1 siRNA. Average of two separate experiments are shown. Only statistical significant differences ($p \leq 0.05$) are reported in the multiplex analysis. Thus the difference is significant in the data shown in the figure.
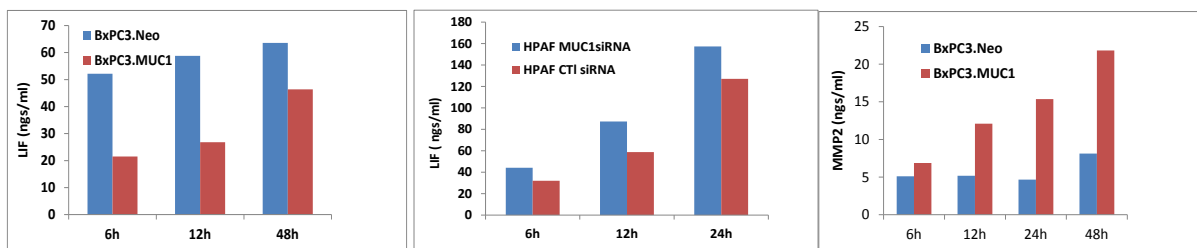
Finally, with regard to targeted drug delivery using the TAB 004 MUC1 monoclonal antibody, we first show that TAB 004 when injected intra peritoneal and intravenously specifically reaches the tumor within 24hours (Figure 10). With iv injection, we observe some reaching the spleen. Therefore, for our future experiments, we plan to inject the antibody intraperitoneal. Second, we show by *in vitro* confocal analysis that TAB 004 is internalized into KCM cells through endocytosis (Figure 11). Thus, we have shown that the blocking peptide is effective *in vivo* and that targeting antibody internalizes in the cells and reaches the tremor bed *in vivo*. Now we are ready to conjugate the blocking peptide to the antibody and start the treatment in the following year.

Figure 10: Localization of TAB004 in mice bearing the MUC1+ve tumor. Mice were injected with biotin-TAB004 (50ugs/mouse) via the ip, it, and iv route. After 24hrs, mice were euthanized and TAB004 binding detected by IHC probing with streptavidin-HRP. Brown staining indicates antibody binding.
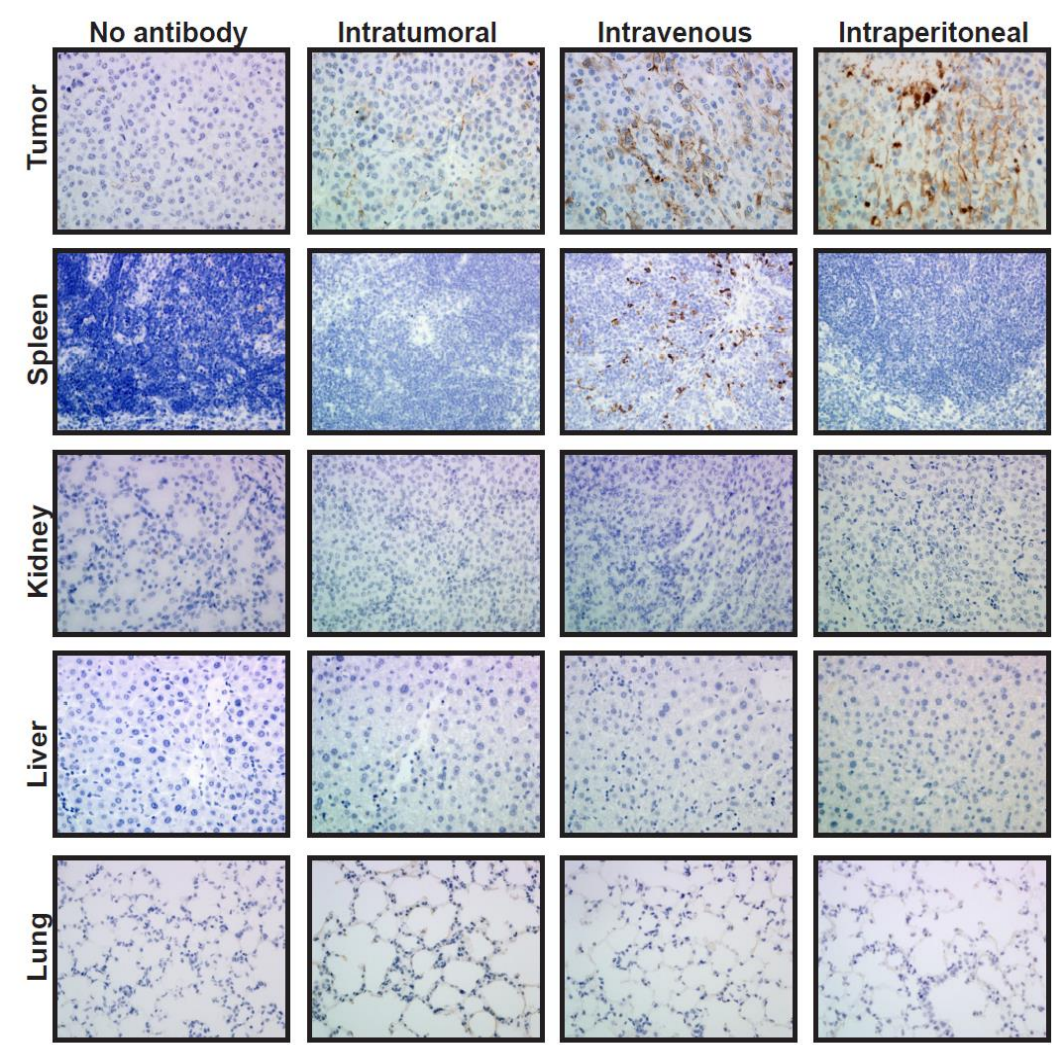
**Figure 11:** TAB004 internalizes in KCM cells. KCM cells were plated on chamber tissue culture slides. Cell surface was labeled green with Cell Light Plasma Membrane-GFP. BacMam 2.0. Lysozyme organelles were labeled red with Lysotracker Deep Red. TAB004 labeled with yellow was incubated with the cells at 1ugs/ml concentration for 2h. Slides were fixed with ProLong Gold Antifade with DAPI and imaged with confocal microscopy.
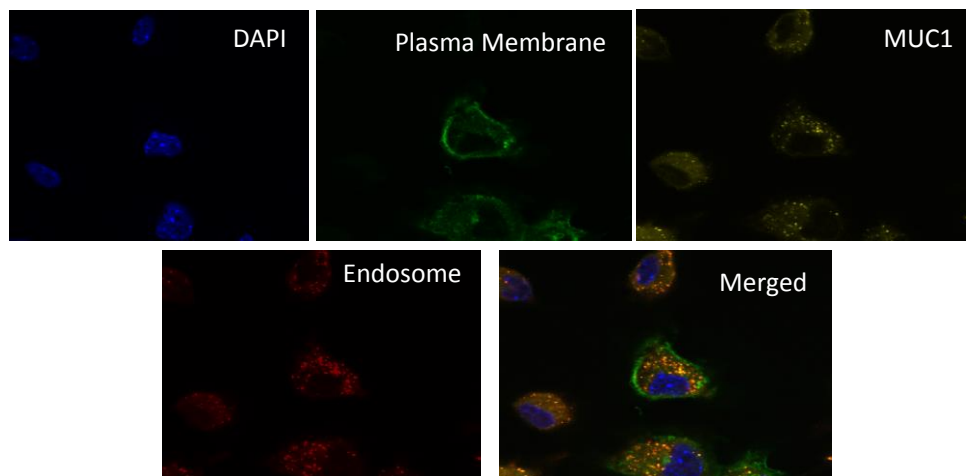
Key Research Accomplishments

Establishment of the zebra fish angiogenic and metastatic assay

MUC1 in pancreatic cancer cells regulate levels of NRP1, VEGFR and angiogenic signaling.

VEGFR, NRP and phosphorylation of VEGFR2 tyrosines is reduced in MUC1-ve tumors

LIF may be one of the underlying factors contributing to inhibition of angiogenesis in MUC1-ve cells

MUC1+ve tumors respond to NRP1 blocking peptide but MUC1-ve cells do not. This suggests that that MUC1 could be a companion diagnostic for anti-angiogenic therapy targeting NRP1.

MUC1 antibody (TAB 004) internalizes in the MUC1+ve tumor cells through endocytosis suggesting that the drug will be internalized when conjugated to TAB 004.

Established the four color confocal images for plasma membrane, lysosomes, MUC1, and DAPI

TAB 004 localizes to the tumor when injected intraperitoneal and intravenous within 24hours.

Reportable Outcomes

- Two manuscripts are in preparation, 1) The effect of NRP1-VEGF signaling axis on MUC1 +ve epithelial tumor cells and 2) The effect of NRP1-VEGF signaling axis in endothelial cells within MUC1+ve tumors. Abstracts are being prepared for submission to the AACR 2014 meeting next month. It will be presented in April of 2014.

- Postdoctoral fellow hired for the project, Graduate students are working on parts of the project and in the future will get publications and degrees out of this work.

- Patient pancreatic cancer serum and tissue collected for future analysis.

- KC, KCM, and KCKO cells are further characterized due to this project

- We plan to submit an RO1 in February of 2014 based on data from this project, especially to understand how LIF plays a role in tumor progression and metastasis

- Development of a novel antibody-anti-angiogenic drug conjugate that can be used in immune competent mouse model of spontaneous PC and can be potentially translated to human clinical trials.

Conclusion

In conclusion, we show that NRP1 may be an excellent target for treating MUC1+ve PDA but not MUC1-ve PDA. The mechanistic role of LIF needs to be further studied. If LIF indeed is the underlying anti-angiogenic signal for MUC1-ve pancreatic cancer, we can envision the potential therapeutic application of LIF1 therapy. It is doubtful that a monotherapy with NRP1 blockade will work and therefore using the TAB 004-conjugated to the drug may show promise due to ADCC caused by the antibody and thereby making the tumors more vulnerable to NRP1 blockade. In the long term, combination therapy with standard of care drug and NRP1 blockade conjugated to TAB 004 or two drugs packaged in nanoparticles and conjugated to the antibody might be highly efficacious for patients with pancreatic cancer.

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